

Hyperhomocysteinemia Evoked by Folate Depletion

Effects on Coronary and Carotid Arterial Function

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Abstract—High circulating concentrations of homocysteine (ie, hyperhomocysteinemia [HHcy]) impair the vascular function of peripheral conduit arteries and arterioles perfusing splanchnic and skeletal muscle regions. The effects of HHcy on coronary resistance vessel function and other indexes of vascular function, ie, arterial permeability and stiffening, are unclear. We tested the hypotheses that HHcy impairs coronary resistance vessel reactivity; increases carotid arterial permeability; and initiates arterial stiffening. Male rats that consumed folate-replete (CON, n=44) or folate-deplete (HHcy, n=48) chow for 4 to 5 weeks had total plasma homocysteine concentrations of 7 ± 2 or 58 ± 4 $\mu\text{mol/L}$, respectively. Maximal acetylcholine-evoked relaxation ($\approx 40\%$ vs $\approx 60\%$) and tension development from baseline in response to nitric oxide synthase inhibition ($\approx 20\%$ vs $\approx 40\%$) were lower (both $P<0.05$) in coronary resistance vessels (≈ 120 μm , internal diameter) isolated from HHcy versus CON animals, respectively, whereas sodium nitroprusside-evoked relaxation and contractile responses to serotonin and potassium chloride were similar between groups. Permeability to 4400 MW and 65 000 MW fluorescently labeled (TRITC) dextran reference macromolecules (quantitative fluorescence microscopy) was $\approx 44\%$ and $\approx 24\%$ greater ($P<0.05$), respectively, in carotid arteries from HHcy versus CON rats. Maximal strain, evaluated by using a vessel elastograph, was less ($\approx 32\%$ vs 42% , $P<0.05$) in carotid arterial segments from HHcy versus CON animals, respectively. Finally, estimates of oxidative (copper-zinc+manganese superoxide dismutase activity) and glycoxidative (pentosidine) stress were elevated ($P<0.05$) in arterial tissue from HHcy versus CON rats. These findings suggest that moderately severe HHcy evoked by folate-depletion impairs endothelium-dependent relaxation of coronary resistance vessels, increases carotid arterial permeability, and initiates arterial stiffening. HHcy may produce these effects by a mechanism associated with increased oxidative and glycoxidative stress. (*Arterioscler Thromb Vasc Biol.* 2002;22:772-780.)

Key Words: coronary resistance artery ■ rat ■ arterial stiffness ■ arterial permeability ■ vascular reactivity

Experimentally confirmed risk factors for cardiovascular disease do not fully account for its widespread prevalence.¹ For example, classic risks including family history, hypercholesterolemia, male sex, physical inactivity, obesity, and smoking are responsible for only 50% to 70% of the actual risk for cardiovascular disease.² Therefore, other variables have come under scrutiny for their potential contribution.

Methionine is an essential amino acid that is released during protein digestion. As methionine is metabolized, the sulfhydryl-containing amino acid homocysteine (Hcy) is formed. Evidence is accumulating that high levels of plasma Hcy are a risk factor for cardiovascular disease.² Hyperhomocysteinemia (HHcy) can result from genetic deficiencies of enzymes required for Hcy metabolism or from nutritional deficits of the vitamins that serve as cosubstrates or cofactors for these enzymes.

HHcy could initiate and/or contribute to cardiovascular risk by impairing vascular function.³ Three indices of arterial function that are compromised to varying degrees in individuals with cardiovascular disease are vascular reactivity,⁴ permeability,⁵ and stiffness.⁶ With specific regard to resistance-sized arteries, HHcy impairs function of vessels perfusing human forearm,^{7,8} rat skeletal muscle,^{9,10} and murine mesentery.¹¹ No studies have examined the effects of HHcy on coronary resistance vessels. This circulation is important because it is the primary regulator of myocardial blood flow.¹² Further, the potential for HHcy to influence arterial permeability has never been investigated, and the effects of HHcy on arterial stiffness are inconclusive.^{13–15} Evaluating these latter two indices of vascular function is relevant clinically because increased permeability could facilitate lipoprotein accumulation in the arterial wall and thus contribute to lesion development and/or severity.⁵ Greater

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arterial stiffness may compromise vasomotor capacity and necessitate increased myocardial oxygen demand.¹⁶

In the present study, we tested the hypotheses that HHcy evoked by folate-depletion 1) impairs coronary resistance vessel reactivity, 2) increases carotid arterial permeability, and 3) promotes arterial stiffening. In addition, tissue markers of oxidative and glycoxidative stress were measured to determine their potential association with HHcy-induced vascular dysfunction.

Methods

Experimental Animals and Diets

All protocols used in this study were approved by the Animal Use and Care Committee at the University of California, Davis, and conformed to guidelines set by the American Physiological Society and Animal Welfare Act. Male Sprague-Dawley rats (50 to 75 g) were housed individually under controlled temperature (23°C) and light conditions (12:12-hour light:dark cycle) and were allowed standard rodent chow and water ad libitum for \approx 1 week after arrival from the breeder. Animals then were divided such that one group received an amino acid defined diet containing 8 g of folate per kg of chow (control animals [CON], $n=44$), while the other consumed a folate-deplete diet (HHcy, $n=48$). Because folate is required to metabolize Hcy to methionine via remethylation, plasma Hcy is elevated in animals that are deficient in folate.¹⁷ An antibiotic (succinylsulfathiazole, 1%) was added to both diets to eradicate intestinal microflora that are capable of synthesizing folate endogenously. Chow consumed by animals in the CON (diet #517814) and HHcy (diet #517777) groups was obtained from Dyets Inc.

General Procedures

After consuming the appropriate diet for 4 to 5 weeks, rats were anesthetized with ketamine (30 to 50 mg/kg, intramuscularly) and xylazine (3 to 5 mg/kg, intramuscularly). The caudal artery then was cannulated to measure arterial pressure and obtain blood samples for later quantification of Hcy and indices of oxidant stress (see Plasma and Tissue Analyses). Both carotid arteries then were isolated and dissected free of surrounding tissue. The right carotid artery was ligated at both ends and cannulated proximally and distally by using a 21-gauge blunt needle. This segment was transferred to a microscope viewing chamber to assess vascular permeability. Segments of the contralateral carotid artery were excised, frozen in liquid nitrogen, and stored at -80°C for later measurement of vascular stiffening and indices of oxidative/glycoxidative stress or placed in ice-cold, oxygenated, normal physiological salt solution (NPSS; pH \approx 7.40) for vascular reactivity experiments. Next, the heart was excised and placed in ice-cold NPSS, and coronary resistance vessels were isolated and used to assess vascular function. Finally, a section of liver and the remaining arterial tree (ie, ascending and descending aorta, right and left iliac and femoral arteries) were immersed in liquid nitrogen and stored at -80°C for later analysis of oxidative or glycoxidative stress. An additional section of liver was obtained and frozen in liquid nitrogen to determine folate concentrations.

Carotid Arterial Permeability

We tested the hypothesis that permeability is greater in carotid arteries from HHcy compared with CON rats. With quantitative fluorescence microscopy, three 10-minute perfusion phases were performed on each vessel. In the initial phase, the artery was perfused (7 mL/min, 100 mm Hg, 37°C , pH \approx 7.4) with clear, non-fluorescent buffer (Krebs-Henseleit solution+1% bovine serum albumin) to measure baseline fluorescence intensity. Second, either 4400 MW dextran molecules (estimated Stokes diameter, 1.4 nm; 42 $\mu\text{g/mL}$ in perfusate) or 65 000 MW dextran molecules (estimated Stokes diameter, 5.7 nm; 42 $\mu\text{g/mL}$ in perfusate) labeled with tetramethylrhodamine isothiocyanate (TRITC; excitation maximum=494 nm, emission maximum=518 nm) were perfused through the arterial lumen and viewed/recorded through an inverted light

microscope. Dextran was used as the reference molecule, because this non-lipid particle does not bind specifically to the artery wall.^{18–21} During this second perfusion phase, there is a rapid increase in intraluminal fluorescence intensity as TRITC-labeled dextran fills the artery lumen. During the third phase, the artery was perfused again with non-fluorescent buffer that washes the TRITC-labeled dextran out of the lumen. The washout phase is analyzed as two distinct processes. The first, rapid washout represents dextran exiting the vessel lumen, whereas the second, slower washout represents dextran exiting the vessel wall. Arterial permeability is estimated by the amount of TRITC-labeled dextran that accumulates in the arterial wall (I_f accumulation). Calculating I_f accumulation involves finding the intersection of tangents drawn to approximate the rapid and slow washout phases. To determine accumulation rate, I_f accumulation is divided by time of perfusion (ie, 10 minutes). Fluorescence values then are converted from millivolts/min to $\text{ng} \cdot \text{cm}^{-2} \cdot \text{min}^{-1}$ by knowing the 1) surface area and 2) lumen volume of the vessel in the photometric window, 3) fluorescence intensity at time 0 (I_0), which occurs at the beginning of dye perfusion, and 4) concentration of dextran in the perfusate. I_f accumulation rates were performed in triplicate for each vessel, and the values were averaged.^{18–21}

Carotid Arterial Elasticity

We tested the hypothesis that vascular distensibility is less in carotid arteries from HHcy versus CON rats. Two stainless steel rods (200- μm outside diameter) were inserted through the lumen of a 1-mm section of carotid artery in a parallel manner while the vessel was immersed in Krebs-Henseleit buffer. One rod was attached to a force transducer, while the other was attached to a motorized controller. This modified vessel myograph, termed an *elastigraph*, allowed the vessel to be stretched in a radial manner at a constant rate until breakage, while vessel tension was recorded via a force transducer. In preparation for each stretch, carotid arterial segments were preconditioned three times at \approx 10% of their maximal load. By using these methods, stress (vessel tension development divided by vessel area) versus strain curves were generated so that maximum strain (ie, strain at vessel breakage), maximal stress (vessel tension at maximum strain), and failure energy (area under the stress-strain curve) could be calculated.^{16,22} Experiments were performed with three 1-mm segments of each carotid artery, and the results were averaged.

Coronary and Carotid Arterial Reactivity

These studies tested the hypothesis that reactivity of coronary microvessels and carotid arteries is impaired in HHcy compared with CON rats. After placing the heart in ice-cold NPSS, the left coronary artery was traced toward the apex with the aid of a dissecting microscope (Leica Stereo Zoom 5). Third-order branches of this artery were isolated, removed, and mounted on a wire-type myograph using methods we have described.^{23,24} After a 30-minute equilibration period, a series of internal circumference–active tension curves was constructed to determine the vessel diameter that evoked the greatest tension development (L_{MAX}) to 100 mmol/L KCl.^{23,24} L_{MAX} was determined for every vessel, and this optimal resting tension was maintained throughout the study.

After a 30-minute equilibration period, five experimental protocols were performed, each separated by 20 to 30 minutes during which the vessel bathing medium was exchanged with NPSS several times. In order, concentration response curves to: 1) a non-receptor mediated vasoconstrictor (KCl; 15 to 100 mmol/L); 2) an endothelium-dependent vasodilator (acetylcholine; 10^{-8} to 10^{-4} mol/L); 3) a receptor mediated vasoconstrictor (5-hydroxytryptamine hydrochloride, serotonin; 10^{-9} to 10^{-5} mol/L); 4) a single dose (10^{-6} mol/L) of N^G -monomethyl-L-arginine (L-NMMA; an inhibitor of nitric oxide synthase); and 5) an endothelium-independent vasodilator (sodium nitroprusside; 10^{-9} to 10^{-5} mol/L) were performed on each vessel. Relaxations to acetylcholine and sodium nitroprusside and constrictions in response to L-NMMA were evaluated from stable tension development in response to 45 mmol/L KCl. Contractile responses are presented as milligrams of tension development, while relaxation

responses are presented as a percentage of KCl-induced precontraction. All five protocols were performed on coronary resistance arteries, whereas only acetylcholine- and sodium nitroprusside-evoked relaxation were evaluated by using carotid arteries. Samples from all buffers and each tissue bath were analyzed frequently for PO_2 , PCO_2 , and pH. Tension data were recorded and processed continuously by a computer through an analog-to-digital interface card (Biopac Systems Inc) that allowed for subsequent off-line quantitative analyses.^{23,24}

NPSS contained (in mmol/L) NaCl 125, KCl 4.7, KH_2PO_4 1.2, MgSO_4 1.2, CaCl_2 2.5, NaHCO_3 18, Na_2EDTA 0.026, and glucose 11.2. Acetylcholine, sodium nitroprusside, L-NMMA (Sigma Chemical), and serotonin (Peninsula Laboratories) were purchased commercially and prepared daily from stock solutions with distilled deionized water.

Plasma and Tissue Analyses

Blood from the caudal artery was collected into prechilled tubes containing EDTA, centrifuged at 2500g for 10 minutes at 4°C, stored at -80°C, and used to quantify total plasma Hcy (tHcy). tHcy refers to the totality of Hcy present after the quantitative reductive cleavage of all disulfide bonds.²⁵ Briefly, tHcy was analyzed by using high-performance liquid chromatography with fluorescence detection.²⁶ This assay quantifies the combination of free reduced homocysteine ($\approx 1\%$ of total), mixed disulfides (≈ 20 to 30% of total), and protein-bound homocysteine (≈ 70 to 80% of total).

Arterial tissue was combined from ≈ 4 animals from the same group and analyzed for 1) copper-zinc+manganese superoxide dismutase activity (Cu-Zn+Mn SOD), an estimate of oxidative stress (kinetic spectrophotometric enzyme assay)²⁷; 2) collagen-associated fluorescence, a general marker of glycoxidative stress (scanning fluorescence spectroscopy)²⁸; and 3) pentosidine, a more specific marker of glycoxidative stress (high-performance liquid chromatography).^{22,29,30}

Sections of liver were obtained to measure thiobarbituric acid reactive substances (TBARS), an estimate of lipid oxidation, by using fluorescence detection.³¹ Procedures implemented to increase the accuracy of this assay included 1) performing the assay on fresh tissue on the day of collection; 2) adding butylated hydroxytoluene to the tissue homogenate to limit TBARS generation during the assay; and 3) using only malondialdehyde that was within the spectrophotometric guidelines. Liver sections also were used to quantify folate with a conventional microbiological assay.³² Tissue protein was determined by using a Bradford reagent (Biorad), and arterial collagen (hydroxyproline) was measured by colorimetric analysis of sample hydrosylates.^{30,33}

Statistical Analyses

Animal and vessel characteristics, indices of arterial permeability and compliance, and all plasma and tissue markers were compared between groups by using an unpaired *t* test.³⁴ Vascular relaxation and constriction responses were compared by using a two-way (dose versus experimental group) repeated-measures ANOVA. Planned comparisons were made at each drug dose to determine whether differences existed between groups. All values are presented as means \pm SE. Statistical significance was accepted when $P < 0.05$.

Results

Animal Characteristics

HHcy rats had higher plasma Hcy concentrations (58 ± 4 vs 7 ± 2 $\mu\text{mol/L}$) and lower liver folate (0.97 ± 0.10 vs 12.10 ± 0.20 μg folate/g liver) and body weight (285 ± 5 vs 333 ± 10 g) than CON animals, respectively. Age (63 ± 2 and 61 ± 1 days), mean arterial blood pressure (74 ± 15 and 82 ± 8 mm Hg), and plasma cysteine concentrations (165 ± 4 and 157 ± 4 $\mu\text{mol/L}$) were similar in HHcy and CON rats, respectively. All animals appeared healthy and robust at the time of study.

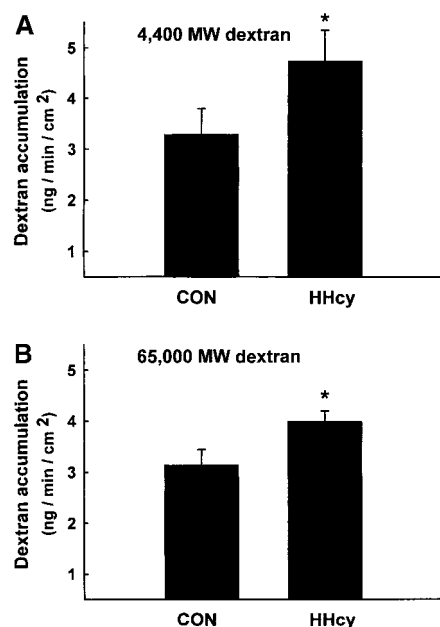


Figure 1. Accumulation of 4400 MW (A) and 65 000 MW (B) dextran reference macromolecules was greater ($P < 0.05$) in carotid arteries from HHcy compared with CON rats. Values are mean \pm SE.

Carotid Arterial Permeability

Accumulation of non-lipid reference molecules within the arterial wall was quantified by using fluorescence microscopy to test the hypothesis that permeability is greater in vessels from HHcy versus CON rats. Compared with vessels from CON rats, those from HHcy animals showed greater accumulation ($\text{ng} \cdot \text{min}^{-1} \text{cm}^2$) of 4400 MW ($\approx 44\%$ greater) and 65 000 MW TRITC-labeled dextran molecules ($\approx 24\%$ greater; Figure 1).

Carotid Arterial Elasticity

Stress versus strain curves were generated with an elastograph to determine whether carotid arteries from HHcy rats are stiffer or less distensible than those from CON animals. We observed a 25% decrease in maximal strain from carotids of HHcy compared with CON rats (Figure 2). Data also indicated a trend ($P = 0.106$) toward decreased failure energy (total area under stress versus strain curve) in vessels from HHcy (25.9 ± 3.0 mN) versus CON (36.4 ± 5.7 mN) rats. Maximal stress (mN/mm^2) was similar in vessels from HHcy (132 ± 13) versus CON (154 ± 10) animals.

Coronary Microvascular and Carotid Arterial Reactivity

Wire-type myographs were used to test the hypotheses that coronary and carotid arterial reactivity are impaired in HHcy versus CON animals. Vessel characteristics were similar between groups for both the coronary and carotid arteries (Table). Acetylcholine produced a dose-dependent relaxation that was impaired in coronary and carotid arteries from HHcy versus CON animals. Relaxation in response to sodium nitroprusside was similar between groups in both vascular segments. These data suggest that endothelium-dependent

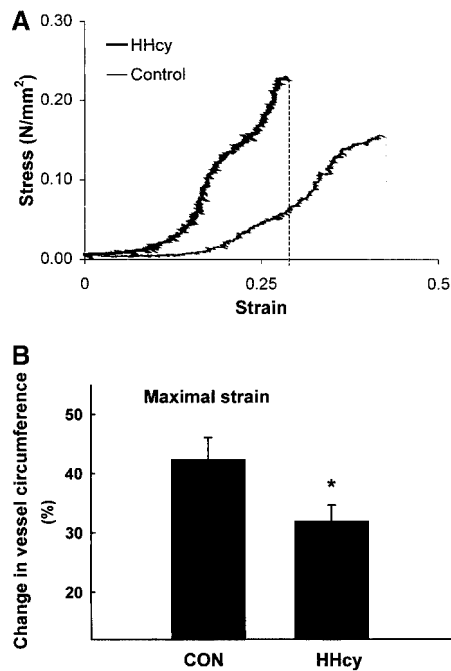


Figure 2. A, Stress versus strain curve of a carotid artery from a CON and HHcy rat. Note that vessel breakage occurs at a lower stress and strain in the HHcy compared with CON vessel. The area under each curve, until the break point, is the failure energy. Group mean data shown in B indicate lower ($P < 0.05$) maximal strain in vessels from HHcy compared with CON animals. Values are mean \pm SE.

function was impaired and endothelium-independent function was unaltered in coronary (Figure 3A and 3B) and carotid arteries (Figure 4) from HHcy versus CON animals. In coronary resistance vessels, L-NMMA-induced tension development (percentage increase from baseline precontraction) was greater in CON ($44 \pm 9\%$) versus HHcy ($23 \pm 7\%$) rats, even though baseline precontractions were similar between

groups (ie, 162 ± 27 and 163 ± 29 mg, respectively). These findings provide an estimate of basal nitric oxide production³⁵ and suggest that it is lower in vessels from HHcy relative to CON animals. Receptor- and non-receptor-mediated contractile responses were similar between groups. Specifically, absolute tension development in response to serotonin and KCl concentration-response curves was not different in coronary resistance vessels from rats that consumed folate-replete or folate-deplete chow. (Figure 3C and 3D) Results were similar when tension development was normalized for vessel length or tension developed at L_{MAX} (data not shown).

Indexes of Glycoxidative and Oxidative Stress

Initially, we quantified collagen-associated fluorescence (CAF), a nonspecific indicator of glycoxidative stress that is thought to result from non-enzymatic glycation. Contrary to our hypothesis, we consistently observed reduced CAF in vessels from HHcy relative to CON rats. Because CAF represents the total amount of fluorescence in vascular collagen digests, we next quantified pentosidine, a highly specific marker of glycoxidative stress,^{22,29,30} using the same tissue hydrosylates. We observed an ≈ 60 -fold increase in pentosidine (pmol pentosidine/ μ g hydroxyproline) in vessels from HHcy (0.932 ± 0.250) versus CON (0.016 ± 0.01) rats, supporting our hypothesis that HHcy evokes glycoxidative stress. Liver TBARS (0.266 ± 0.117 vs 0.152 ± 0.035 nmol malondialdehyde/mg protein) and arterial tissue Cu-Zn+Mn SOD activity (146 ± 9 vs 108 ± 3 mol \cdot min⁻¹ \cdot mg⁻¹ protein) also were greater in HHcy versus CON animals, respectively. Hydroxyproline (μ g/cm² of carotid artery) was similar in arterial tissue from CON (140 ± 10) and HHcy rats (135 ± 14).

Discussion

We tested the hypothesis that arterial function is impaired in rats that were made hyperhomocysteinemic by consuming a folate-deplete diet. Our findings indicate that HHcy 1) impairs

Characteristics of Carotid Arteries and Coronary Resistance Vessels

	CON	HHcy
Coronary resistance arteries		
Internal diameter, baseline, μ m	128 ± 13	111 ± 4
Internal diameter, L_{MAX} , μ m	277 ± 14	250 ± 12
%Increase in internal diameter, baseline to L_{MAX}	124 ± 12	128 ± 12
Tension developed at L_{MAX} , mg	217 ± 9	182 ± 6
%Precontraction for acetylcholine dose-response	84 ± 2	87 ± 4
%Precontraction for sodium nitroprusside dose-response	96 ± 8	93 ± 7
%Precontraction before adding N^G -monomethyl-L-arginine	75 ± 6	75 ± 8
Carotid arteries		
Internal diameter, baseline, μ m	541 ± 16	544 ± 20
Internal diameter, L_{MAX} , μ m	848 ± 20	787 ± 19
%Increase in internal diameter, baseline to L_{MAX}	59 ± 5	47 ± 6
Tension developed at L_{MAX} , mg	530 ± 30	540 ± 40
%Precontraction for acetylcholine dose-response	87 ± 4	93 ± 3
%Precontraction for sodium nitroprusside dose-response	86 ± 5	88 ± 10

Values are mean \pm SEM.

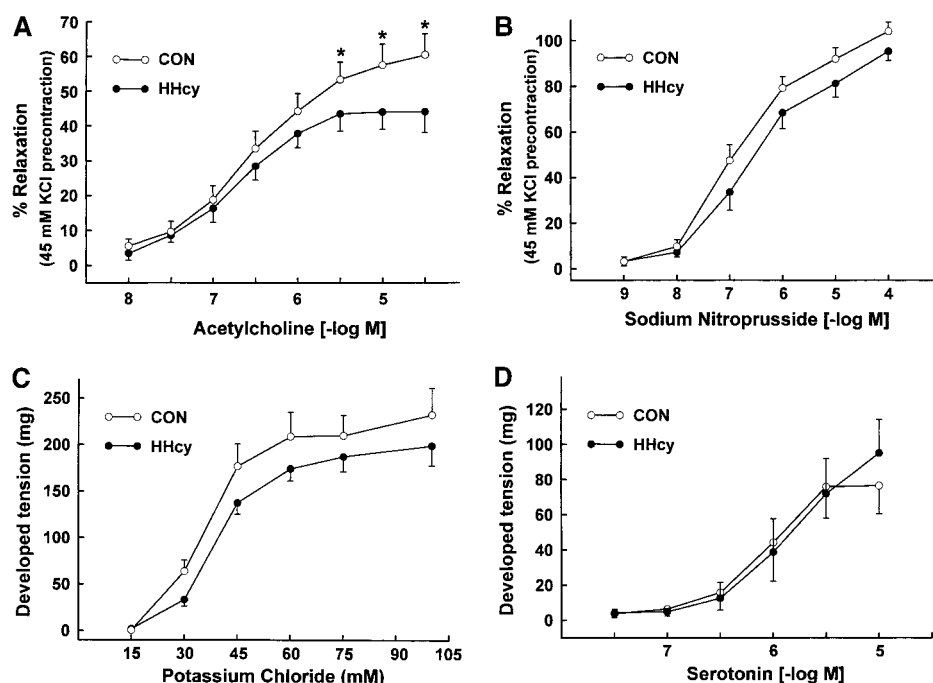


Figure 3. Vascular reactivity experiments show that acetylcholine-evoked relaxation was less ($*P < 0.05$, A) in coronary resistance arteries from HHcy compared with CON rats, while sodium nitroprusside-evoked relaxation (B) was similar between groups. Tension development in response to potassium chloride (C) and serotonin (D) was similar between groups. Values are mean \pm SE.

acetylcholine-evoked relaxation in coronary resistance vessels and carotid arteries, 2) increases estimates of vascular permeability in carotid arteries, and 3) decreases maximal arterial distensibility in carotid arteries. Further, estimates of oxidative (Cu-Zn+Mn SOD) and glycoxidative (pentosidine) stress were

elevated in arterial tissue from HHcy versus CON animals. Collectively, these findings support the hypothesis that vascular function is impaired by moderately severe HHcy and may be associated with increased oxidative and glycoxidative stress. While these data confirm previous reports regarding the influence of HHcy on conduit artery (ie, aorta, femoral, carotid, and brachial artery) function,^{11,36–41} new information is presented concerning coronary microvascular reactivity and permeability and stiffness of conduit arteries.

Reactivity of Coronary Resistance Arteries

Our study is the first to investigate the influence of moderately severe HHcy on isolated coronary resistance vessel reactivity. This circulation is important, because these arteries provide the primary resistance to myocardial blood flow.¹² Moreover, because a heterogeneity exists among vessels of various sizes and from different vascular beds regarding reactivity to similar agents,^{12,42} the effects of HHcy cannot necessarily be extrapolated from one region to another. For instance, HHcy (tHcy ≈ 9 vs $4 \mu\text{mol/L}$) impaired endothelium-dependent dilation of murine mesenteric arterioles to a much greater extent than aortic rings, even though vessels were obtained from the same animals.¹¹ Therefore, results from studies examining the effects of HHcy on large conductance arteries cannot be extrapolated to small resistance vessels.

We observed that acetylcholine-evoked relaxation is impaired, whereas sodium nitroprusside-evoked relaxation is unchanged, in coronary resistance vessels from HHcy versus CON rats (tHcy ≈ 58 vs $7 \mu\text{mol/L}$, respectively). These findings suggest that endothelium-dependent relaxation is compromised, whereas nitric oxide/cGMP-dependent intra-

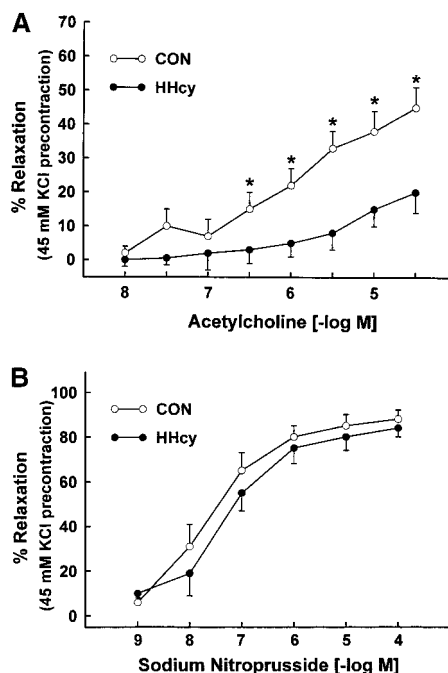


Figure 4. Vascular reactivity experiments show that acetylcholine-evoked relaxation was less ($*P < 0.05$, A) in carotid arteries from HHcy compared with CON rats, while sodium nitroprusside-evoked relaxation (B) was similar between groups. Values are mean \pm SE.

cellular signaling pathways within vascular smooth muscle are unchanged by HHcy produced by folate depletion. Our results may have been different had another endothelium-dependent agent and/or preconstrictor substance been used. For example, the potential contribution to vasorelaxation from endothelium-derived hyperpolarizing factor (EDHF) cannot be evaluated in vessels that have been precontracted by using KCl. In this regard, EDHF has been shown to contribute to acetylcholine-evoked relaxation in canine coronary microvessels⁴³ and rat mesenteric arteries.⁴⁴ We tried several alternate endothelium-dependent vasorelaxing substances and preconstricting agents in preliminary studies^{23,24} but there was a lack of reproducibility and unstable tension development, respectively, rendering them unacceptable. In any case, we refer to our assessment of endothelial function as acetylcholine-evoked vasorelaxation and believe that nitric oxide release evoked by muscarinic receptor stimulation is largely responsible for the observed vasorelaxation. Evidence supporting this is that acetylcholine-evoked relaxation in KCl-precontracted rat coronary microvessels is abolished by atropine (10^{-6} mol/L) or L-NMMA (10^{-6} mol/L).²³ Nevertheless, because coronary microvessels and carotid arteries (see below) were precontracted with KCl, any role that EDHF may have in acetylcholine-evoked relaxation in rats with HHcy cannot be determined in the present study.

In addition to stimulated (ie, acetylcholine-evoked) release of nitric oxide being blunted by HHcy, we observed $\approx 50\%$ less L-NMMA-evoked constriction (ie, an estimate of basal nitric oxide release)³⁵ in coronary resistance vessels from these animals compared with CON rats. Although we believe that the generated tension was in response to L-NMMA-induced inhibition of endothelial cell nitric oxide synthase, other sources of this enzyme may have been blocked. For instance, recent findings indicate that nitric oxide synthase is present in vascular smooth muscle of bovine carotid arteries and can effect physiological contractile responses.⁴⁵ Another consideration is that functional estimates of basal nitric oxide production in the present study are specific to vessels precontracted with KCl. Therefore, results may have been different had another L-arginine analogue and/or preconstrictor agent been used.

We also assessed tension development of coronary microvessels in response to receptor-mediated (ie, serotonin) and non-receptor mediated (ie, KCl) contractile agents. Serotonin was chosen for study because it is an important regulator of vascular tone and is released in significant quantities when platelets aggregate in response to a local thrombotic event.⁴⁶ KCl-induced tension development was assessed because it occurs via activation of voltage-gated calcium channels and thus represents a contractile mechanism that is receptor-independent. Contractile responses to these two agents were similar between groups. Therefore, it appears that reduced stimulated (ie, acetylcholine-evoked) and basal (ie, L-NMMA-evoked) nitric oxide production observed in vessels from HHcy animals was not severe enough to result in exaggerated contractile responses to serotonin and KCl.

Reactivity, Permeability, and Stiffness of Conduit Arteries

Impaired endothelial function of conduit arteries has been demonstrated in animal and human models of HHcy. For example, endothelium-dependent relaxation was compromised in aortic rings from CBS+/- mice fed standard (tHcy ≈ 9 $\mu\text{mol/L}$),¹¹ methionine-enriched (tHcy ≈ 24 $\mu\text{mol/L}$),⁴¹ or folate-reduced chow (tHcy ≈ 25 $\mu\text{mol/L}$),³⁹ and carotid arteries from monkeys that consumed methionine-enriched/folic acid-reduced chow (tHcy ≈ 11 $\mu\text{mol/L}$).³⁶ The tHcy control values from these studies were ≈ 4 , 6, 6, and 4 $\mu\text{mol/L}$, respectively. Further, flow-mediated dilation was blunted in femoral arteries from monkeys,³⁶ brachial and femoral arteries from patients with homocystinuria (tHcy ≈ 63 vs 13 $\mu\text{mol/L}$),³⁸ and the brachial artery of healthy individuals after ingesting methionine (tHcy ≈ 25 vs 10 $\mu\text{mol/L}$) or homocysteine (tHcy ≈ 53 vs 10 $\mu\text{mol/L}$) orally.⁴⁰ Our results confirm and extend these findings to include isolated carotid arteries from rats with moderately severe HHcy evoked by folate depletion (tHcy ≈ 58 vs 7 $\mu\text{mol/L}$).

We also determined whether arterial permeability is increased in carotid arteries from HHcy compared with CON rats. Strong rationale exists for posing this question. First, HHcy has been shown to exert oxidant stress,⁴⁷ a finding that was confirmed in the present study by our observations that liver TBARS and arterial tissue Cu-Zn+Mn SOD and pentosidine were greater in HHcy than in CON animals. Second, we have reported that arterial permeability is increased by oxidant stress, and these changes are attenuated by several anti-oxidants.¹⁹ Finally, HHcy has been shown by some laboratories to increase atherosclerotic lesion progression.^{48–51} To address whether HHcy evokes oxidant stress to an extent whereby arterial permeability is increased, real-time measurements of dextran accumulation were made by using methods whereby flow rate, hydrostatic pressure, pH, temperature, and superfusate and perfusate compositions were controlled to simulate physiological conditions. We observed that this estimate of arterial permeability, ie, the accumulation rate of 4400 and 65 000 MW dextran reference molecules, was greater in arteries obtained from HHcy compared with CON animals, thus supporting our hypothesis. These data, taken together with findings that HHcy initiates a cascade of inflammatory mediators,^{48,52} may partly explain why some investigators have shown that atherosclerotic lesion development is enhanced by HHcy.

Another index of vascular function ie, arterial stiffness, was estimated in carotid arteries from HHcy and CON rats. Increased arterial stiffness is observed in patients with atherosclerosis, diabetes, and hypertension.⁵³ Further, vascular stiffening may be a cause or consequence of impaired reactivity and is associated with increased arterial permeability. A consensus regarding the influence of HHcy on estimates of vascular stiffness is lacking. In this regard, HHcy has been shown to correlate strongly,¹³ marginally,¹⁵ or not at all¹⁴ with arterial stiffness in experiments that used a variety of methods to evaluate stiffness and evoke increases in Hcy. In the present study, vascular stiffening was estimated by quantifying the passive elastic properties of carotid arteries by using an elastograph. We observed lower maximal strain in

carotid arteries from HHcy versus CON animals, indicating that HHcy causes vessels to become less maximally distensible.⁵⁴ Additionally, there was a trend toward decreased failure energy in carotid arteries from HHcy rats, suggesting that carotid arteries have reduced "elastic" capacity.⁵⁵ More severe biomechanical alterations may have occurred had the duration of HHcy been extended beyond 4 weeks.

Increased arterial stiffening and/or vascular permeability may be caused by the accumulation of advanced glycation end-products (AGEs) that occurs during non-enzymatic glycation within the vascular wall.²² In the present study, we sought to determine whether HHcy causes an increase in glycoxidation, thus resulting in the accumulation of AGEs. This is particularly important with regard to HHcy-induced vascular dysfunction, because increased expression of an AGE receptor recently was reported in mice with HHcy.⁴⁸ We observed a dramatic increase in pentosidine, a standard and specific in vivo biomarker of glycoxidative damage,^{22,29,30} in arterial tissue from HHcy relative to CON animals. These data, taken together with our findings that estimates of oxidative stress (eg, liver TBARS and arterial Cu-Zn + Mn SOD) are elevated in rats with HHcy evoked by folate depletion, suggest that vascular dysfunction is due, at least in part, to increased oxidative and glycoxidative stress.

Concern regarding our interpretation of oxidant stress may be raised. Specifically, elevated Cu-Zn+Mn SOD activity could reflect increased oxidant stress and/or increased antioxidant capacity. We believe that Cu-Zn+Mn SOD activity was greater in arterial tissue from HHcy versus CON rats in response to oxidant stress evoked by HHcy. In this regard, exposure of animals to high-oxygen environments or to compounds that increase intracellular superoxide anion generation (eg, methylene blue) results in increased SOD activity.⁵⁶ Further, the genetic response to superoxide in bacteria includes an induction of SOD.⁵⁷ This information, taken together with findings that superoxide anion production is greater in aorta from CBS-deficient mice,¹¹ lead us to believe that elevated arterial Cu-Zn+Mn SOD was functioning as an antioxidant defense mechanism to correct/compensate for an imbalance in the redox state (eg, increased superoxide anion) created by HHcy. Although this explanation is feasible, it cannot be made with certainty because we did not measure superoxide anion production directly.

Past studies have produced HHcy by 1) acute Hcy ingestion; 2) acute and chronic methionine ingestion; 3) heterozygous deletion of the gene encoding CBS in mice (ie, CBS+/-); 4) folate-restriction/depletion; and 5) combinations of 2, 3, and/or 4. We elevated Hcy concentrations to a moderately severe (58 ± 4 μ M) and pathophysiologically relevant degree^{58,59} by feeding rats folate-deficient chow for 4 to 5 weeks. Because folate is required by the two main pathways responsible for Hcy metabolism, ie, the remethylation pathway and the transsulfuration pathway, plasma Hcy concentrations increase in rats that consume folate-deficient chow.¹⁷ Although this method is extremely reproducible and reliable, it does have limitations. For example, some studies,⁶⁰⁻⁶³ but not all,^{48,64} indicate that low folate levels contribute to cardiovascular disease in a manner that is independent of the ability of low folate to

elevate Hcy. Interestingly, endothelial dysfunction of aortic rings was observed in CBS+/- mice rendered hyperhomocysteinemic via methionine supplementation compared with those that consumed standard chow, even though both groups possessed normal liver folate concentrations.⁴¹ Whereas these results suggest that vascular reactivity is compromised by HHcy per se rather than by its combination with folate depletion, it should be noted that CBS+/- mice fed a folate-deficient diet developed aortic vascular dysfunction more quickly than CBS+/- mice supplemented with methionine. Further, folate deficiency has been shown to directly impair endothelial nitric oxide production⁸ and may have contributed to vascular dysfunction observed in the present study. Because of this, caution should be used when extrapolating our findings beyond the experimental setting of moderately severe HHcy evoked by folate depletion.

In summary, we observed that moderately severe HHcy produced by folate depletion 1) impairs endothelium-dependent relaxation of coronary microvessels and carotid arteries, 2) increases carotid arterial permeability to reference macromolecules, 3) decreases maximal carotid arterial distensibility, and 4) elevates indices of oxidative and glycoxidative stress. These findings provide new information regarding the multiple methods whereby HHcy may contribute to cardiovascular disease.

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